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Short communication

Increased genome instability is not accompanied by sensitivity to DNA damaging agents in aged yeast cells

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ABSTRACT

The budding yeast *Saccharomyces cerevisiae* divides asymmetrically, producing a new daughter cell from the original mother cell. While daughter cells are born with a full lifespan, a mother cell ages with each cell division and can only generate on average 25 daughter cells before dying. Aged yeast cells exhibit genomic instability, which is also a hallmark of human aging. However, it is unclear how this genomic instability contributes to aging. To shed light on this issue, we investigated endogenous DNA damage in *S. cerevisiae* during replicative aging and tested for age-dependent sensitivity to exogenous DNA damaging agents. Using live-cell imaging in a microfluidic device, we show that aging yeast cells display an increase in spontaneous Rad52 foci, a marker of endogenous DNA damage. Strikingly, this elevated DNA damage is not accompanied by increased sensitivity of aged yeast cells to genotoxic agents nor by global changes in the proteome or transcriptome that would indicate a specific "DNA damage signature". These results indicate that DNA repair proficiency is not compromised in aged yeast cells, suggesting that yeast replicative aging and age-associated genomic instability is likely not a consequence of an inability to repair DNA damage.

1. Introduction

Budding yeast *Saccharomyces cerevisiae* cells divide asymmetrically. Each original "mother" cell buds off a new "daughter" cell. While the daughter cell lineage is immortal, the mother lineage is finite. A mother cell can only divide on average 25 times before it senesces and dies. Age-promoting factors are thought to be preferentially segregated to the mother cell during cell division, allowing the daughter cell to be born with a full lifespan, but causing progressive aging of the mother cell [reviewed in 1]. This form of yeast aging, termed replicative aging, has been used to model aging of mitotically dividing human cells, such as stem cells.

Genome instability is a hallmark of human aging [2]. Numerous premature aging diseases, such as Werner syndrome and Cockayne syndrome, are caused by mutations in DNA repair genes (WRN for Werner syndrome, CSA and CSB for Cockayne syndrome) [3,4]. Similarly, many yeast DNA repair mutants have decreased replicative lifespans [5,6]. In particular, in addition to a short lifespan, deletion of *SGS1* (the yeast homolog of WRN) causes premature sterility, nucleolar fragmentation, and accumulation of extrachromosomal ribosomal DNA circles (ERCs), which are characteristics of aged yeast cells [7,8]. Aged yeast cells also exhibit a dramatic increase in loss of heterozygosity (LOH) [9], and the stability of the ribosomal DNA (rDNA) locus decreases with age [8,10,11]. In addition, a recent study found elevated levels of DNA double-strand breaks (DSBs), transfer of mitochondrial DNA into the nuclear genome, large-scale chromosomal alterations, and retrotransposition during yeast replicative aging [12].

What remains unclear is whether DNA repair capacity is compromised in old yeast cells, or if the observed increase in genomic instability can be explained by elevated endogenous genotoxic stress experienced by aging cells. Here, we analyzed the behavior of DNA damage response factors in the recently published yeast aging transcriptome and proteome [13], and examined the occurrence of endogenous DNA damage as well as the response to genotoxic treatments in replicatively aging yeast cells.

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Table 1
Yeast strains used in this study.

Strain name	Relevant genotype	Source
UCC8773	*MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 hoΔ::Pscw11-cre-EDB78-NatMX loxP-CDC20-intron-loxP-HphMX loxP-UBC9-loxP-LEU2*	[45]
DNY61	*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 RAD52-GFP::HIS3MX NUP49-mCherry:Ca-URA3*	This study

2. Materials and methods

2.1. Yeast strains and growth conditions

Standard yeast media and growth conditions were used [14,15]. Yeast strains used in this study were constructed in the BY4741 genetic background [16] and are listed in Table 1. To obtain DNY61, the *NUP49-mCherry* allele was introduced into the *RAD52-GFP* strain from the GFP collection [17] by crossing and tetrad dissection.

2.2. Bioinformatic analysis

"DNA damage signatures" were defined as sets of genes/proteins changing significantly in abundance upon exposure to genotoxic agents, as published: 0.1% methyl methansulfonate (MMS), 1 h [18], 0.02% MMS, 2 h [19], 170 Gy ionizing radiation (IR), 30 min [19], 0.03% MMS, 2 h [20], 0.2 M hydroxyurea (HU), 2 h [20], 0.2 M HU, 160 min [21]. Age-related abundance changes of proteins and transcripts were taken from [13], as the fold change (log2) occurring when comparing successive time points of replicative aging to young cells. The last time point of the time series, 72 h of cultivation corresponding to an average of 24 divisions, was used as the prototypical "aged" sample for analysis. All data processing was done in the R scripting environment [22]. To search for a general DNA damage signature that may be present in aged cells, all genes changing by a specific DNA damaging agent were assessed for their behavior in the aging protein/transcript dataset. The distribution of changes these DNA damage signature genes underwent with age was compared to the general distribution of all changes occurring with age. A significant difference detected between these distributions (Wilcoxon signed-rank test, $p < 0.01$) was indicative that a specific DNA damage signature was occurring with age (Fig. 1A). Next, we looked specifically at the significance of overlap between (1) the transcripts/proteins in the DNA damage signatures and (2) the transcripts/proteins changing at least twofold with age (Fig. 1B) using Fisher's exact test (significance at $p < 0.01$) in a pairwise fashion. Only those genes overlapping between the respective DNA damage study and aging dataset were considered. Visualization of the overlap was done using the venneuler package [23].

2.3. Microfluidics and fluorescence microscopy

Cell cultures for microfluidic experiments were grown in SD-complete medium supplemented with 0.1% BSA. The presence of BSA coats the PDMS microfluidic device, which facilitates the efficient removal of daughter cells by fluid flow. The experiments were performed using ALCATRAS 2, as described in [24]. A total flow rate of 4 µl/min (provided by two syringes) was used to provide the cells trapped inside the device with nutrients and prevent overgrowth of the device. All experiments were performed at 30 °C. Images were acquired using a DeltaVision Elite imaging system (Applied Precision (GE), Issaquah, WA, USA) composed of an inverted microscope (IX-71; Olympus) equipped with a Plan Apo 60X (1.4 NA) oil immersion objective, InsightSSITM Solid State Illumination, excitation and emission filters for FITC and A594, ultimate focus and a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ, USA).

Bright-field images were taken every 20 min for up to 80 h. Fluorescent images were taken at the beginning of the experiment and 2, 5, 10, 15, 20, 30, 40, 50 and 60 h into the experiment (for Fig. 2B), or at 0 and 15 h into the experiment (for Fig. 2C), or in 10-min intervals over a 60-min time window starting 0 or 15 h into the experiment (for Fig. 2D and E). Stacks of 9 images with 0.5 µm spacing were taken at an exposure time of 50 ms for FITC and 400 ms (at 50% intensity) for A594. Only cells that stayed in the device for a whole lifespan (for Fig. 2B) or until the end of the experiment (for Fig. 2C), and had lived for more than 3 divisions, were included in the analysis. Processing of all images was performed using Fiji (ImageJ, National Institutes of Health).

2.4. Preparation of young and old yeast cells

To obtain cultures of young and aged cells, an overnight culture was diluted 100-fold and grown for 4–5 h at 30 °C to obtain exponentially growing cells. The culture was then adjusted to a concentration of 4×10^4 cells/ml, and estradiol was added to a final concentration of 1 µM to induce the Mother Enrichment Program (MEP) [25]. Half of the culture was analyzed just before (for UV treatment) or immediately after (for IR, MMS, and HU treatment) induction by estradiol (young cells), while the other half was incubated for 20 h shaking at 30 °C (old cells).

2.5. Genotoxic treatments

Young and old cells were mock-treated or treated with UV, IR, MMS, or HU at the indicated doses. Cells were diluted 10-fold prior to plating (to dilute estradiol, MMS, and HU). All experiments were performed with 4 plates (technical replicates) per dose/time point. Plating volumes were adjusted for young and old cells to obtain ~200 colony forming units per plate. For UV treatment, plated cells were irradiated with UVC using a light-box (UVC 500 Ultraviolet Crosslinker, Amersham Biosciences, Germany). Plates were wrapped in aluminum foil right after irradiation and kept in the dark during incubation to prevent photoreactivation. For IR treatment, cells were irradiated, while still in the liquid culture, with gamma rays from a cesium-137 source. IR doses of 30, 60, 90 and 150 Gy correspond to a treatment time of 18, 36, 54 and 90 min, respectively. Untreated controls for each time point were also analyzed. For MMS and HU treatment, liquid cultures were incubated with the indicated MMS or HU concentrations for 20 min or 2 h, respectively.

3. Results and discussion

3.1. The transcriptome and proteome of aging yeast cells are not characterized by a clear DNA damage response

To investigate the presence of a "DNA damage signature" or to detect possible global alterations in the DNA damage response during replicative aging, we aimed to see if the transcriptome and proteome of aging yeast cells [13] resemble those of cells treated with genotoxic agents [18–21]. Upon treatment with DNA damaging agents, Jelinsky et al. [18] and Gasch et al. [19] identified transcriptional responses, and Chong et al. [21] and Tkach et al. [20] identified responses at the proteome level. We used these lists of differentially expressed transcripts and proteins as examples of DNA damage signatures to compare to the transcriptome and proteome of replicatively aged yeast cells [13]. The DNA damage transcriptomes and proteomes had been generated under six different conditions that induce DNA lesions, namely variable exposures to methyl methanesulfonate (MMS), ionizing radiation (IR), or hydroxyurea (HU). A comparison of the collective changes observed resulting from these treatments with the changes observed in aging showed little overlap (Fig. 1A). Specifically, while two of the twelve DNA damage signatures show a statistically sig-

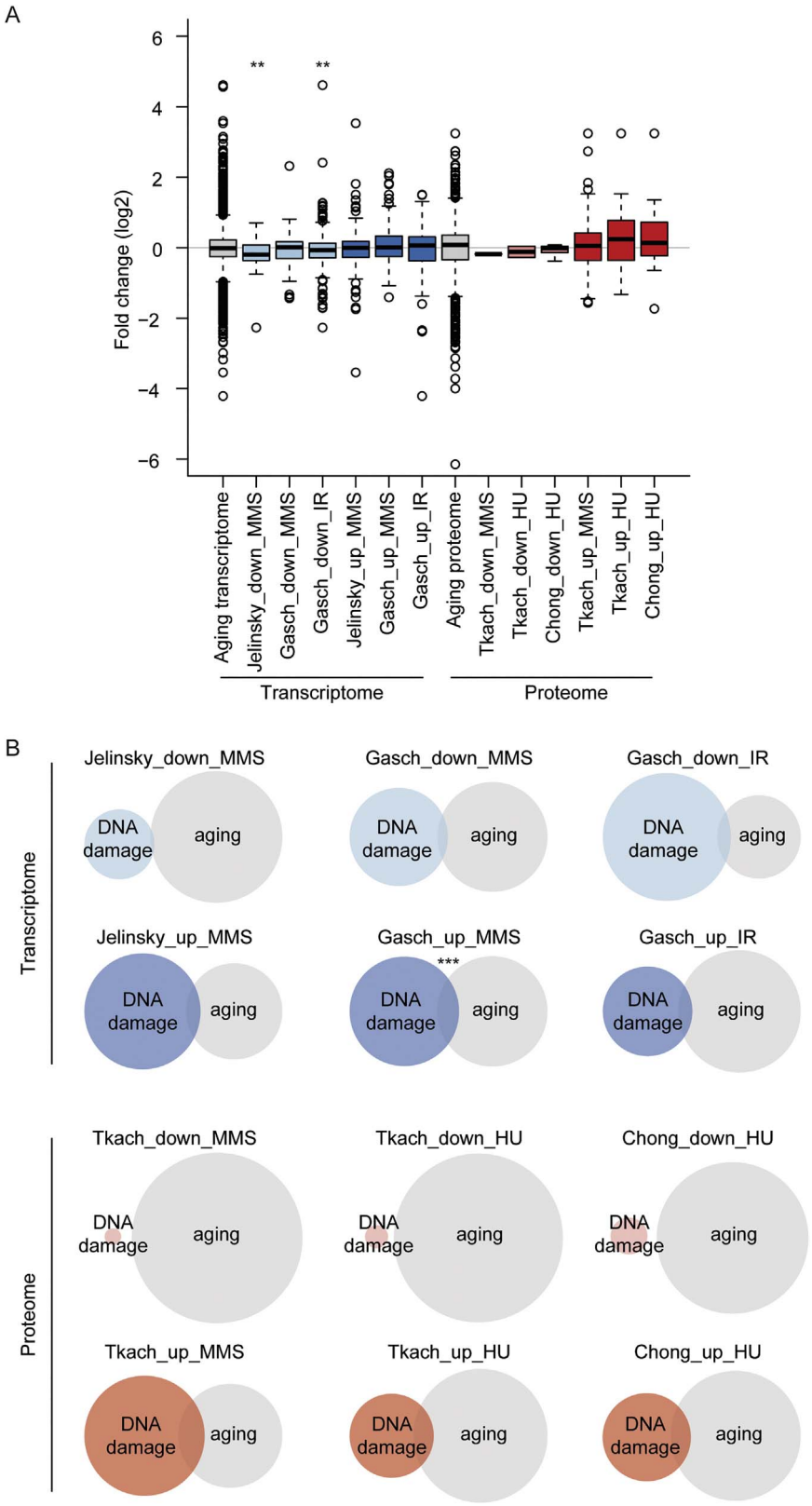

Fig. 1. Yeast aging is not characterized by a clear DNA damage transcriptional or proteomic signature.Gene lists either up or down regulated at either the transcriptional [18,19] or proteomic [20,21] levels when treated with DNA damaging agents (MMS: methyl methanesulfonate; IR: ionizing radiation; HU: hydroxyurea) were compared to the proteome and transcriptome of old (72 h of cultivation, ~24 divisions) yeast cells [13]. (A) The gene lists of the DNA damage signatures do not significantly change in aged yeast, as would be expected if a DNA damage response were occurring. Only two (Jelinsky_down_MMS, Gasch_down_IR) out of 12 signatures were found to be significantly different in expression with age (** $p < 0.01$, Wilcoxon signed-rank test). Box-and-whisker plots are presented as follows: the thick black line within the box is the median of the data, the box extends to the upper and lower quartile of the dataset (i.e. to include 25% of the data above and below the median, respectively), whiskers (dashed lines) represent up to 1.5 times the upper or lower quartiles, and circles represent outliers. (B) The gene lists of the DNA damage signatures were compared to only those genes found to change twofold with age, at either the transcriptional or proteomic levels, for significant overlap. Sizes of Venn diagram circles are scaled to largest dataset in each pairwise comparison. Only one (Gasch_up_MMS) signature out of the 12 was found enriched in the genes changing twofold with age (*** $p < 0.001$, Fisher's exact test), suggesting no clear enrichment of DNA damage signatures with age.

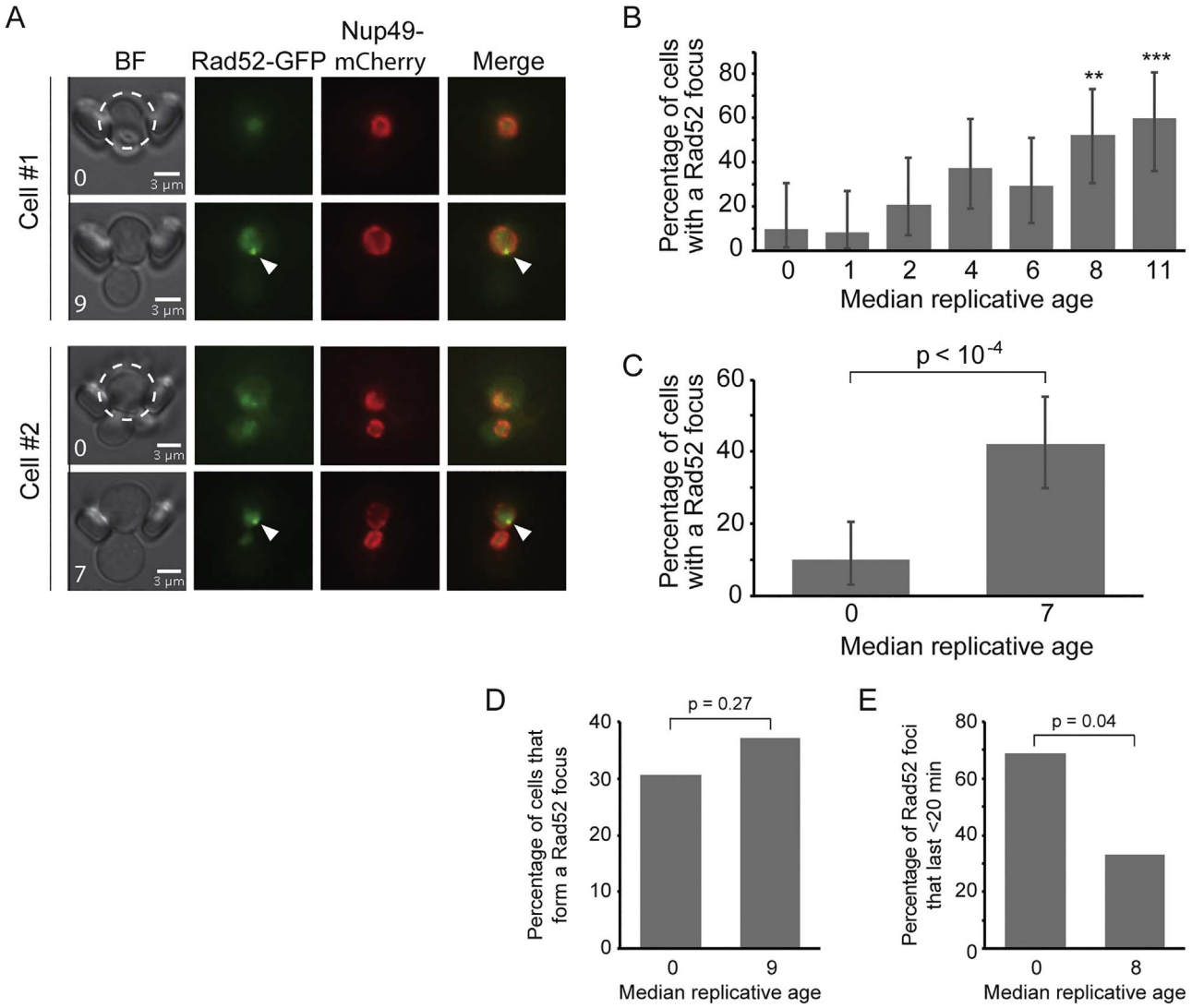

Fig. 2. Increased spontaneous Rad52 foci in aging yeast cells.Yeast cells expressing Rad52-GFP and Nup49-mCherry (a marker of the nuclear periphery) were loaded into the ALCATRAS microfluidic device and imaged at different time points during replicative aging. Fluorescence images were scored for the presence of a Rad52-GFP focus, and bright-field images (BF) were used to count the number of divisions for each mother cell (replicative age). (A) Two examples of mother cells displaying a Rad52 focus (white arrow) at the indicate replicative age. The white dashed circles in the BF images indicate the mother cells at the first time point. (B) Fraction of cells displaying a Rad52-GFP focus at the indicated replicative ages. Images were taken at 0 h, 2 h, 5 h, 10 h, 15 h, 20 h, 30 h, 40 h, 50 h and 60 h (number of cells analyzed: 24). Error bars indicate exact binomial 95% confidence intervals. Significant differences with respect to age 0 are marked by asterisks (Fisher's exact test; ** $p < 0.01$; *** $p < 0.001$). (C) Fraction of cells displaying a Rad52-GFP focus at the indicated replicative ages. Images were taken at 0 h and 15 h (number of cells analyzed: 64). Error bars indicate exact binomial 95% confidence intervals. Statistical significance was assessed with Fisher's exact test, and the p-value is indicated. (D) Fraction of young and aged cells that form a Rad52 focus during a 60-min window (number of cells analyzed: 127 young, 43 old), and (E) the fraction of those foci that are processed within 20 min are shown (number of foci analyzed: 29 young, 12 old). Statistical significance was assessed with Fisher's exact test, and the p-values are indicated.

nificant shift in expression with age ($p < 0.01$), the majority, 10 out of 12, did not, suggesting that the yeast transcriptome and proteome are not characterized by a clear DNA damage response with aging (Fig. 1A).

To further explore this possibility, we examined only those transcripts or proteins that change twofold with age [13], and compared how the DNA damage signature sets overlap with these genes. We again found no consensus towards an enrichment of DNA damage signatures with aging; while one DNA damage signature at the proteome level was found to be statistically enriched in proteins changing twofold with age ($p < 0.01$), 11 of the 12 remaining DNA damage signatures were not significantly enriched (Fig. 1B). Together these findings suggest that a classic DNA damage response is not overwhelmingly present in yeast aging at either the transcriptional or proteomic levels.

While specific and isolated transcripts and proteins related to the DNA damage response may significantly change in abundance with age (outliers in Fig. 1A, shown in Fig. S1 and File S1 (in the online version at DOI: http://dx.doi.org/10.1016/j.dnarep.2017.03.005), and as described in [26]), and aging may have a slight enrichment in changes occurring in response to certain genotoxic treatments, there is no global trend towards a DNA damage response with aging. We base this conclusion on the observations that 10 out of 12 of the DNA damage signatures did not show a statistically significant shift in expression with age (Fig. 1A), and the specific genes changing at least twofold in expression with age did not show any enrichment in 11 out of the 12 DNA damage signatures (Fig. 1B). Therefore, if a DNA damage signature is indeed occurring with age, it is heavily masked by other age-related changes. It is also important to note that the aging dataset was obtained using a prototrophic strain closely related to S288c [27] that was biotinylated and bound to streptavidin-conjugated irons beads and cultured in a column with a constant flow of fresh medium, while the DNA damage datasets were obtained using auxotrophic strains of an S228c-related [18,20,21] or W303 background [19], grown in more standard laboratory growth conditions. These differences may complicate the comparison between the aging and DNA damage datasets, so

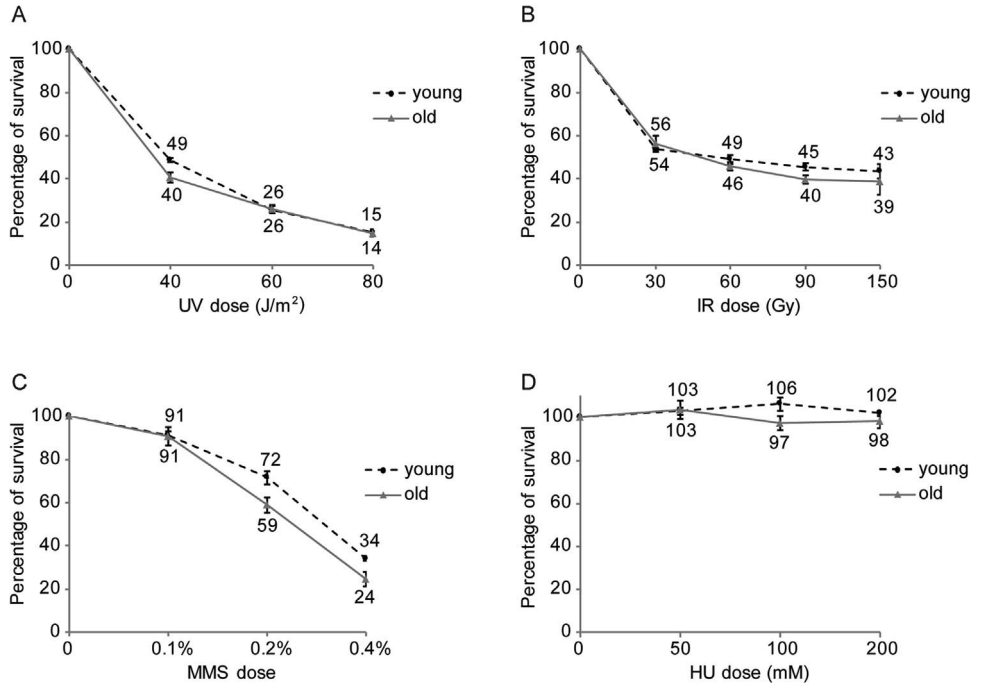

Fig. 3. Aged yeast cells do not display increased DNA damage sensitivity.Survival curves for young (t = 0 h) and old (t = 20 h, corresponding to age ~13) MEP cells exposed to UV (A), IR (B), MMS (C), and HU (D) at the indicated doses. The duration of the MMS and HU treatments were 20 min and 2 h, respectively. Mean values from three independent experiments are plotted. Error bars represent standard error.

we sought a more direct method to assess genome instability in aging cells.

3.2. Age-dependent increase in spontaneous Rad52 foci

In order to more directly assess the occurrence of spontaneous DNA damage, we decided to monitor the formation of Rad52-GFP foci in living cells during aging. Rad52, a recombination protein involved in DSB repair, forms a clearly detectable nuclear focus in response to DNA damage [28,29]. Spontaneous Rad52 foci occur in absence of genotoxic treatment in 5–10% of logarithmically growing yeast cells and are considered markers of endogenous DNA damage [30].

To monitor Rad52-GFP focus formation in aging yeast cells, we used the recently developed ALCATRAS microfluidic device [24]. Fluorescence images of individual mother cells trapped in the ALCATRAS chip were taken at different time points throughout lifespan and cells were scored for the presence of Rad52-GFP foci (examples are shown in Fig. 2A). Bright-field images were taken every 20 min to monitor the replicative age of each individual cell during the experiment. Rad52 foci are detectable in less than 10% of very young cells (median RLS = 0–1), in agreement with published observations in exponentially growing cultures [30]. This fraction progressively increases as cells age, where up to 60% of the cells display a Rad52 focus at age 11 (Fig. 2B). To rule out the possibility that the increase in Rad52 foci was an artefact due to phototoxicity as cells are repeatedly imaged during the course of the experiment, we noted that no difference in focus formation is detectable between the first two fluorescence images taken (Fig. 2B, replicative age 0 and 1, corresponding to time points 0 and 2 h, respectively), indicating that there is no evidence of phototoxicity-induced Rad52 foci by imaging cells at two time points. Thus, we repeated the experiment taking only two time points, corresponding to ages 0 and 7, and could still observe a significant fourfold increase in Rad52 foci (Fig. 2C), confirming the results of our initial experiment. Our findings are in agreement with the observed increase in rDNA instability, ERC formation, and LOH in old cells, since these processes are recombination-dependent [8–11]. Moreover, Rad52 is required for DSB repair, and DSBs are also elevated in aged cells [12].

The age-dependent increase in the percentage of cells with a Rad52 focus could be due to more cells forming foci or to foci lasting longer. To distinguish between these possibilities, we imaged cells at 10-min intervals over a 60-min time window either immediately (young) or 15 h (aged) after loading the cells into the ALCATRAS device. In cells that did not have a focus at the start of the 60-min window, roughly one-third of the cells formed foci during this time window, both when the cells were young or aged (Fig. 2D). In contrast, in the young cells, but not the aged cells, these foci are mostly resolved within 20 min (Fig. 2E). The longer duration of Rad52 foci in aged cells likely reflects a delay in kinetics of DNA repair.

3.3. No evidence of DNA damage sensitivity in aged yeast cells

Since the increase in Rad52 foci with age suggests possible defects in DNA repair, we assessed the sensitivity of young and old cells to genotoxic treatments. To obtain a population of old cells, we took advantage of the Mother Enrichment Program (MEP), a genetic system which restricts proliferation of daughter cells after induction with estradiol, thus allowing the study of a cohort of aging mother cells [25]. In this system, the Cre recombinase is fused to an estradiol-binding domain and expressed from a daughter-specific promoter; introduction of estradiol causes the fusion protein to be transported into the nucleus where Cre disrupts two essential genes, *UBC9* and *CDC20*, in daughter cells [25]. Yeast cells were analyzed immediately (young) or 20 h after (old, corresponding approximately to age 13) induction of the MEP by estradiol. As a validation of our experimental setup, we were able to observe an increase in the frequency of *petite* colony formation in old cells, as previously reported [31] (Fig. S2 in the online version at DOI: http://dx.doi.org/10.1016/j.dnarep.2017.03.005). We then determined the sensitivity of young an old cells to UV, IR, MMS, and HU, which induce different DNA lesions [32,33]. Cells lacking Rad52 are sensitive to all four genotoxic agents, especially IR and MMS [34–36]. Based on the observed increase in Rad52 focus formation (Fig. 2), together with the elevated DNA damage and genomic instability reported for old cells [8–12], we expected that aged cells should be less efficient in responding to exogenous genotoxic stress. However, we

did not observe any significant increase in the sensitivity of old cells compared to young cells for any of the genotoxic treatments tested (Fig. 3). Although surprising, many mutant strains with elevated levels of spontaneous Rad52 foci [30,37] have no reported sensitivity to genotoxic agents [35,36,38–41], indicating that increased Rad52 focus formation is not always accompanied by DNA damage sensitivity.

4. Conclusions

Our results suggest that, despite evidence of elevated DNA damage during yeast replicative aging ([12] and Fig. 2), the transcriptome and proteome of aged cells do not show a classic DNA damage response (Fig. 1), likely because aging is a complex phenomenon affecting many biological processes. Furthermore, we find that aged yeast cells are not hypersensitive to four different genotoxic agents (Fig. 3). One explanation could be that endogenous damage is being repaired, but as some DNA repair mechanisms are intrinsically mutagenic [42], we see enhanced genomic instability as cells age. Alternatively, or additionally, as previously suggested [9,43], aging may cause a shift in repair pathway utilization, with error-free DNA repair predominant in young cells and the use of mutagenic DNA repair mechanisms increasing as cells age. If this is the case, old cells might still be able to withstand endogenous or exogenous genotoxic insults, but at the expense of faithful maintenance of the genome. Our data suggest that yeast replicative aging and age-associated genomic instability are not due to an inability to repair DNA damage, although we cannot exclude that defects in these mechanisms arise at late stages of yeast lifespan, beyond the latest time points examined in our study.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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